STAT3 and STAT5 signaling in normal and leukemic hematopoietic cells

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Chapter 7

Downregulation of GATA1 uncouples STAT5-induced erythroid differentiation from stem/progenitor cell proliferation

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Summary

Previously, we have shown that overexpression of an activated mutant of STAT5 induces erythropoiesis, impaired myelopoiesis and an increase in long-term proliferation of human hematopoietic stem/progenitor cells. Since GATA1 is a key transcription factor involved in erythropoiesis, the involvement of GATA1 in STAT5-induced phenotypes was studied by knocking down GATA1 using a lentiviral shRNA approach. $CD34^+$ cordblood cells were double transduced with a conditionally active STAT5 mutant and a lentiviral vector expressing a short hairpin against GATA1. Erythropoiesis was completely abolished in the absence of GATA1, indicating that STAT5-induced erythropoiesis is GATA1-dependent. Furthermore, the impaired myelopoiesis in STAT5-transduced cells was restored by GATA1 knockdown. Interestingly, early cobblestone formation was only modestly affected and long-term growth of STAT5-positive cells was increased in the absence of GATA1, whereby high progenitor numbers were maintained. Thus, GATA1 downregulation allowed the dissection of STAT5-induced differentiation phenotypes from the effects on long-term expansion of stem/progenitor cells. Gene expression profiling allowed the identification of GATA1-dependent and GATA1-independent STAT5 target genes, and these studies revealed that a number of proliferation-related genes were upregulated by STAT5 independent of GATA1, whereas a number of erythroid differentiation related genes were found to be GATA1 as well as STAT5 dependent.

Introduction

Signal Transducer and Activator of Transcription (STAT) 5 is a member of a family of transcription factors that is comprised of seven genes (STAT1 to -6, with STAT5 being encoded by two genes, STAT5A and –B). The pathways mediated by these transcription factors are involved in many processes in hematopoietic cells, including regulation of proliferation, anti-apoptosis, differentiation and self-renewal.[1,2] STAT transcription factors contain a conserved tyrosine residue in their SH2 domain, which upon phosphorylation, gives rise to hetero- or homo dimerization (or tetramerization[3]) with another STAT molecule, leading to nuclear translocation. Constitutively activated STAT5 has been found in blast cells of up to 70 % of AML cases.[4] Besides its function as a transcription factor, tyrosine phosphorylated STAT5 has also been described to interact with other partners to induce PI3K activity, thereby contributing to leukemic transformation.[5] STAT transcription factors can be activated by growth factors via JAK kinases, present at the cytoplasmic domains of growth factor receptors, as well as by intrinsic kinase activity of certain membrane receptors or cytoplasmic tyrosine kinases.[6]

STAT5 is expressed in a large variety of hematopoietic cells, and involved in the signal transduction of many different growth factors and cytokines. Early acting cytokines like FLT-3 ligand (FLT-3L), Thrombopoietin (TPO) and Stem Cell Factor (SCF) all mediate at least part of their signals through STAT5.[7-9] During erythropoiesis, STAT5 is critically involved in the signal transduction mediated by Erythropoietin (EPO).[10] Furthermore, signals induced by myeloid growth factors like Interleukin (IL)-3, IL-5, G-CSF, M-CSF and GM,CSF are all, at least in part, mediated by STAT5.[1,11,12] Loss-of-function studies in mice have revealed a critical role for STAT5 in the maintenance of hematopoiesis as shown by a reduced repopulation ability in $STAT5A^{-/-}$, $STAT5B^{-/-}$ double knockout mice.[13-16] We have shown that knockdown of STAT5 in a $CD34^{+}$ cells from cord blood resulted in reduced stem cell and progenitor frequencies,[17] whereas over expression of active STAT5A promoted stem cell self renewal.[18,19] STAT5 plays an important role in differentiation towards the erythropoietic lineage by up regulating anti-apoptotic pathways via Bcl-XL[10,20] but a more direct role in inducing erythroid differentiation is likely to exist as well.[18,19,21-23]

GATA1 is a transcription factor, originally identified by the ability to bind a distinct motif present in promoter and enhancer sequences of a variety of erythroid genes. This motif, WGATAR, was shown to be critical in the regulation of these genes.[24]

Apart from GATA1, five other members of the GATA family have been identified (named GATA2 to -6). Of the six GATA proteins, GATA1 to -3 are expressed in hematopoietic cells, whereas GATA4 to -6 are expressed in non-hematopoietic tissues like the heart, gut, lung and liver.[25] GATA1 and GATA2 are critically involved in erythroid and megakaryocytic cell development.[26] It has been shown in transgenic mice carrying the GATA2 regulatory domain fused to GFP (and therefore recapitulating the endogenous GATA2 expression) that GATA2 is detected very early in hematopoietic development.[27] Analogous experiments with a lacZ reporter under the control of the GATA1 regulatory elements showed expression at somewhat later stages of erythroid development.[28] Gene knockout studies have indicated that homozygous deletion of both GATA1 and GATA2 is embryonically lethal at day 10.5 to 12.5.[29,30] Studies performed in GATA1 knockout embryonic stem cells have revealed that GATA1-null cells did not contribute to red blood cell formation, while white blood cell formation in chimeric animals was normal.[31] Furthermore, GATA1-null cells were arrested at the proerythroblast stage in *in vitro* colony formation assays, underscoring the essential role of GATA1 in erythroid development.[31] GATA1low mice, lacking a DNA hypersensitive site in the GATA1 promoter and therefore expressing only 10 to 25 % of normal GATA1 levels, are thrombocytopenic but display initially normal hematocrit levels.[32] After 12 months, these mice however develop anemia accompanied by myelofibrosis.[32]
We have previously shown that overexpression of active STAT5A results in a profound burst of erythropoiesis, accompanied by impaired myelopoiesis and enhanced self-renewal and long-term expansion.[18,19] The underlying mechanisms have not been elucidated yet. Since GATA1 has been shown to be essential for erythropoiesis, we decided to investigate the possible dependence of STAT5-induced phenotypes on GATA1 by a lentiviral GATA1 knockdown approach. We observed that depletion of GATA1 allowed the dissection of STAT5-induced differentiation phenotypes from the effects of STAT5 on long-term expansion of human hematopoietic stem/progenitor cells. This provided us with a tool to further study the molecular mechanisms and genes involved in these processes.

Materials and methods

Cell culture and viral transductions

Neonatal cord blood was obtained from healthy full-term pregnancies from the Obstetrics departments of the Martini Hospital and University Medical Center in Groningen, The Netherlands, after informed consent. The protocol was approved by the Medical Ethical Committee of the UMCG. $CD34^+$ cells were isolated with the use of a hematopoietic progenitor isolation kit from Miltenyi Biotech according to the manufacturer's instructions. The pMSCV-STAT5AwtER-IRES-tNGFR retroviral vector was described previously.[19] An RNAi-resistant GATA1 expressing vector was made by introduction of silent mutations at the RNAi target site by PCR-mediated site directed mutagenesis in the cDNA of human GATA1. The resulting RNAi-resistant GATA1 cDNA was fused to a myc tag and cloned into the pMSCV-IRES-EGFP vector in which the EGFP was replaced with cDNA encoding for dTomato, kindly provided by B. Giepmans (dept. of Cell Biology, University of Groningen, Groningen, the Netherlands), resulting in pMSCV-mutGATA1myc-IRES-dTomato. Stable PG13 high titer retroviral producer cell lines were generated as described previously.[19] GATA1 was knocked down by lentiviral transduction of $CD34^+$ cord blood cells using a modified version of the pLVUT-tTRKRAB-shGATA1 vector, which was a kind gift of D. Trono (School of Life Sciences, Ecole Polytechnique Federale de Lausanne (EPFL), Lausanne, Switzerland), in which the IRES-KRAB and TetO sequences were deleted, resulting in constitutive expression of the short hairpin against GATA1. A control vector was made by cloning a hairpin against firefly luciferase into the pLVUT vector. Lentiviral particles were made by transient transfection of 293T cells with the lentiviral expression vectors as described previously.[33] Isolated $CD34^+$ cells were cultured for 48 hours in serum free medium (HPGM, Lonza, Breda, the Netherlands) containing 100 ng/ml each of SCF, TPO and FLT-3 ligand and subsequently transduced with the retro- and lentiviral particles in three consecutive rounds as described previously.[33]

Stromal Coculture assays, Colony Forming Cell (CFC) assays

MS5 cocultures were performed as described previously.[19] Half of the cultures was removed weekly and analyzed by FACS or sorted to perform CFC assays or cytospins. CFC assays were performed in MethoCult H4230 (StemCell

Technologies, Grenoble, France) supplemented with 20 ng/ml Interleukin-3 (IL3), 20 ng/ml IL-6, 20 ng/ml G-CSF, 20 ng/ml c-Kit ligand (KL) and 1 U/ml EPO. 10^3-10^4 cells were plated in methylcellulose per plate in duplicate. Cultures were scored using a Leica DM-IL inverted microscope (Leica Microsystems, Rijswijk, the Netherlands) at a total magnification of 40x.

mRNA analysis

Total RNA was isolated using the RNeasy kit from Qiagen (Qiagen, Venlo, The Netherlands) according to the manufacturer's recommendations. For real-time RT-PCR, cDNA was prepared and amplified using iQ SYBR Green supermix (Bio-Rad, Veenendaal, the Netherlands) in a MyIQ thermocycler (Bio-Rad), and quantified using MyIQ software (Bio-Rad). HPRT, Ribosomal Protein Like (RPL) 27 and RPL30 expression levels were used to normalize between samples and to calculate relative expression levels.[34] Primers and conditions are available on request. Genome-wide expression analysis was performed on Illumina (Illumina, Inc., San Diego, CA) BeadChip Arrays Sentrix Human-6 (46k probesets). Typically, 0.5-1 µg of RNA combined from three independent transduction experiments was used in labeling reactions and hybridization with the arrays according to the manufacturer's instructions. Data was analyzed using the BeadStudio v3 Gene Expression Module (Illumina, Inc.) and Genespring (Agilent, Amstelveen, The Netherlands).

Flow cytometry analysis

Antibodies against CD11b, CD14, CD15, CD34, CD38, CD45RA, CD71 and CD123 were obtained from Beckton Dickinson (Breda, the Netherlands). Anti NGFR-APC was obtained from Miltenyi Biotec (Utrecht, the Netherlands). GlycophorinA-PE was obtained from Dako (Heverlee, Belgium). Cells were incubated with antibodies at 4°C for 30 min. All FACS analyses were performed on an LSRII flowcytometer (Becton Dickinson) and data was analyzed using WinList 3D (Topsham, USA) or FlowJo (Tree Star, Oregon, USA) software.

Immunoblotting and cytospins

Sorted cells were boiled in Laemmli sample buffer for 5 min prior to separation on 12% SDS-polyacrylamide gels. Proteins were transferred to PVDF membrane

(Millipore, Etten Leur, The Netherlands) by semidry electroblotting. Membranes were blocked in Odyssey blocking buffer (Westburg, Leusden, the Netherlands) prior to incubation with antibodies. Binding of antibodies was detected by incubating with Alexa680 or IRDye800 labeled secondary antibodies (Invitrogen, Breda, the Netherlands) and scanning of the membrane on an Odyssey infrared scanner (Li-Cor Biosciences, Lincoln, NE, USA). Antibodies against STAT5 (C17), GATA1 (N6) and Actin (C4) were obtained from Santa Cruz (Santa Cruz Biotech, Santa Cruz, CA, USA) and were used in dilutions of 1:1000. May-Grünwald Giemsa staining was used to stain cytospins. Cytospin preparations were evaluated and photographed using a Leica DM3000 microscope (Leica Microsystems, Rijswijk, the Netherlands) equipped with a Leica DFC420C digital camera at a total magnification of 400x. Images were cropped and reverted to grayscale using Corel Photopaint 12 (Corel Corp., Ottawa, Canada) software.

Results

STAT5-induced erythroid differentiation is GATA1- dependent

Previously, we observed that persistent activation of STAT5 in human CB $CD34^+$ cells resulted in long-term self-renewal and expansion, while erythropoiesis was induced (summarized in Fig.1A).[18,19] To assess whether GATA1 would be dispensable for the increased erythropoiesis induced by STAT5, GATA1 was knocked down in CB $CD34^+$ cells expressing a 4 hydroxytamoxifen (4-OHT)-activatable form of STAT5 (Fig.1B).[19] Both GATA1 mRNA levels (Fig.1C) as well as GATA1 protein levels (Fig.1D) were efficiently downregulated in GATA1 shRNA-transduced cells. STAT5A activation did not change GATA1 mRNA or protein expression levels (Fig.1C and D).

Next, the short hairpin against GATA1 was expressed in $CD34^+$ cord blood cells together with STAT5-ER and the cells were cultured on MS5 bone marrow stromal cells. After ten days of culture, part of the suspension cells were harvested and stained for Glycophorin A (GPA) expression as a measure for erythroid differentiation. As indicated in figure 1E, activation of STAT5 induced a strong erythroid differentiation within 10 days of MS5 coculture (Fig.1E upper panel, 88%

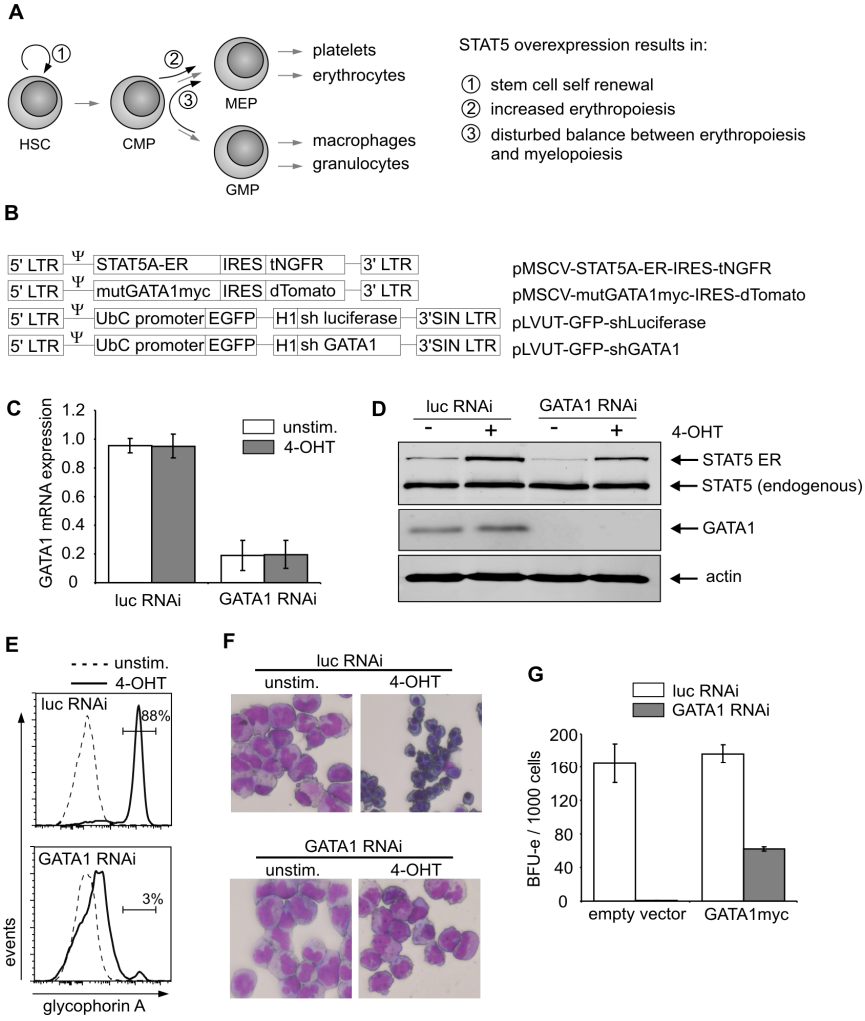

Figure 1: STAT5 induced erythroid differentiation is GATA1 dependent. (A) Schematic overview of previously obtained results caused by active STAT5 over expression. (B) Retro- and lentiviral constructs used in this study. (C) Quantitative RT-PCR results of cord blood CD34$^+$ cells that were double transduced with the STAT5A-ER vector in combination with the indicated lentiviral RNAi constructs. Cells were left untreated or stimulated for 24 hours with 4-OHT, sorted and RNA was extracted. GATA1 mRNA expression was calculated after normalization against expression of HPRT, RPL27 and RPL30 mRNA. The shown graph represents the mean of three independent experiments. (D) Western blot of sorted cells, transduced and treated as in C. Extracts were blotted against STAT5, GATA1 and actin to show equal loading. (E) Representative FACS histograms of glycophorin A expression on cells transduced with STAT5A-ER and the indicated lentiviral vectors expressing short hairpins against luciferase or GATA1. Cells were cultured on MS5 stromal cells, left untreated or stimulated with 4-OHT and analysed for Glycophorin A expression after 10 days. (F) May-Grünwald-Giemsa (MGG)-stained cytospin preparations of suspension cells of cultures described in E and harvested after 2 weeks. (G) BFU-E numbers of methylcellulose culture of cord blood cells, transduced with lentiviral vectors for expression of short hairpins against luciferase and GATA1, together with an empty expression vector or a vector expressing RNAi resistant, myc tagged GATA1. 1000 double transduced cells were sorted one day after transduction directly into CFC medium. BFU-E colonies were counted after 2 weeks.

GPA$^+$), which was strongly reduced by expression of the GATA1-specific hairpin (Fig.1E lower panel, 3% GPA$^+$). Erythroid differentiation was also monitored morphologically. As depicted in figure 1F and 2A, STAT5A activation resulted in the appearance erythroid cells at various stages of commitment, including erythroblasts, orthochromatic normoblasts with distinct pyknotic nuclei to the enucleated erythrocyte stage. These erythroid cells were virtually absent after knockdown of GATA1 (Fig.1F, lower panels). Furthermore, the efficacy as well as the specificity of the GATA1 knockdown was investigated by expressing the short hairpin in the absence and presence of an expression vector for mutant GATA1. This mutant GATA1 was genetically modified to be resistant to the used shRNA by introduction of silent mutations at the recognition site of the hairpin. The formation of BFU-e colonies was assayed after transduction of cord blood CD34$^+$ cells with the shRNA vectors as well as an empty vector control or mutant GATA1 expressing vector and subsequent sorting of the double transduced cells. It is shown in figure 1G that expression of the short hairpin against GATA1 resulted in a robust reduction of the number of colonies, which could be partially rescued by expression of shRNA resistant GATA1.

Restored balance between erythroid versus myeloid differentiation upon GATA1 downmodulation in cells expressing activated STAT5

Since the balance between erythroid and myeloid differentiation was disturbed in CB CD34$^+$ cells expressing activated STAT5A (fig.1A),[18,19] we questioned whether downmodulation of GATA1 would modulate myelopoiesis. CB CD34$^+$ cells were transduced with STAT5-ER together with vectors containing the short hairpin against GATA1 or a control shRNA vector. The cells were cultured on MS5 stromal cells and the phenotype of the suspension cells resulting from these cultures was determined for a period of five weeks. Each week, the double transduced cells from the suspension fraction were sorted, analyzed morphologically by performing MGG staining on cytospin preparations, and analyzed phenotypically by flow cytometry for the presence of erythroid and myeloid markers. Figure 2A shows cytospin preparations of a representative experiment (left panel) as well as the expression of the myeloid marker CD11b and the erythroid marker GPA at the indicated time points (right panel). Activation of STAT5 by 4-OHT clearly resulted in a relative increase in erythroid cells in the first three weeks, as judged by enumeration of erythroid cells in the MGG-stained cytospins. The increase in erythroid

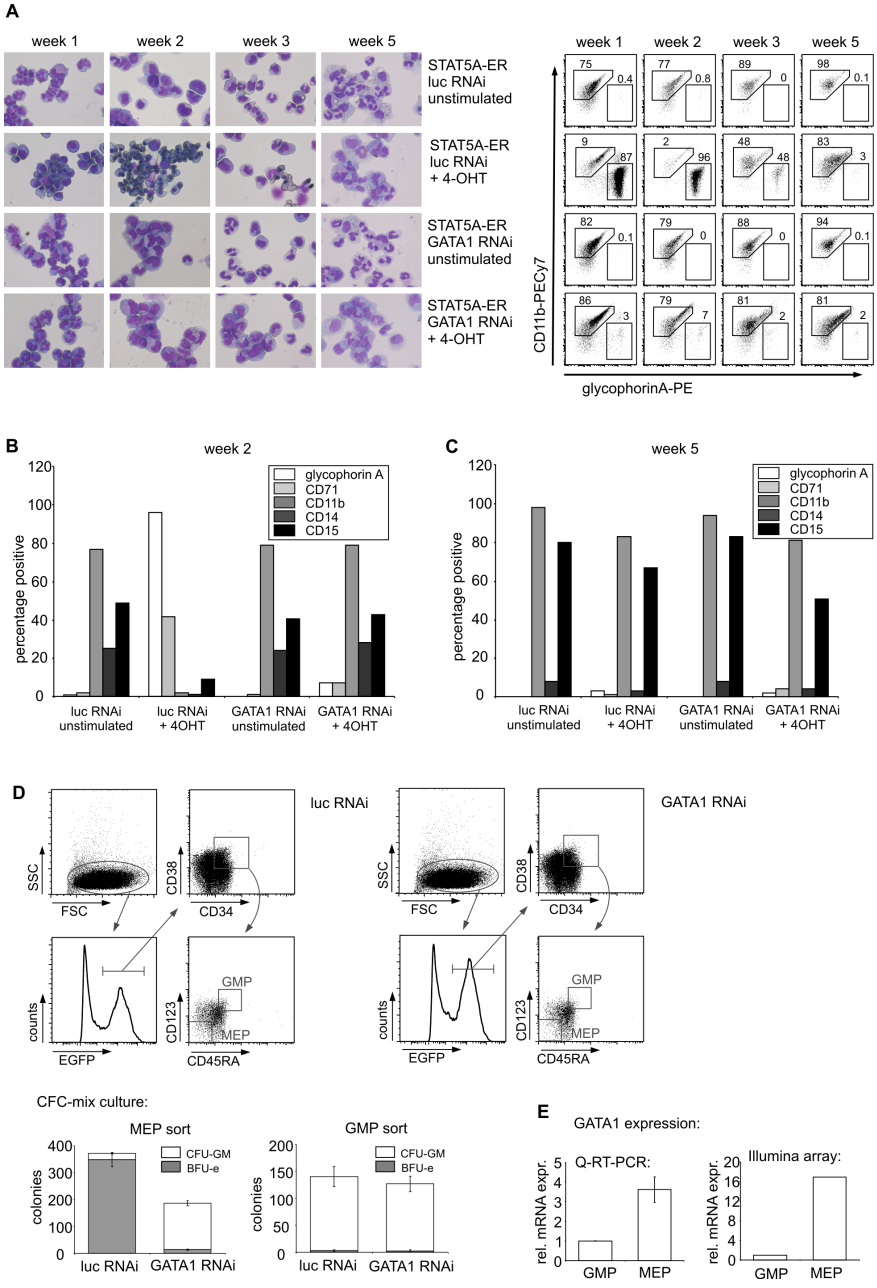

Figure 2: STAT5 induced block in myeloid differentiation is reversed after GATA1 depletion. (A) CD34^{+} cord blood cells were transduced with STAT5A-ER and the indicated lentiviral RNAi constructs. After transduction, cells were cultured on MS5 stromal cells and either left untreated or stimulated with 4-OHT. Each week, half of the medium was removed and double transduced cells were sorted and cytospin preparations were made (left panel). The same cells were analysed by flow cytometry for the expression of CD11b and Glycophorin A at week 1, 2, 3 and 5. Representative FACS plots are shown in

the right panel with percentage positive cells indicated in the plots. (B) FACS analysis of the expression of Glycophorin A, CD11b, CD14 (monocyte/macrophage), CD15 (granulocyte) and CD71 (transferrin receptor) of cells cultured and stimulated as in A at week 2. (C) FACS data as in B from week 5. In B and C one representative experiment of three experiments performed is shown. (D) $CD34^+$ cord blood cells were transduced with the indicated lentiviral shRNA constructs. One day after transduction, the cells were stained for CD34, CD38, CD45RA and CD123 and transduced (EGFP positive) cells were sorted according to the shown gates to obtain MEP and GMP progenitor populations. The sorted cells were cultured in methylcellulose medium to enumerate the number of BFU-Es and GFU-GMs. After two weeks, progenitors were scored. (E) GATA1 expression in GMPs and MEPs as determined by Q-RT-PCR and Illumina arrays.

differentiation relative to myeloid differentiation was also observed by FACS, whereby the expression of CD11b was dramatically decreased in the 4-OHT-stimulated cultures (from 75%, 77% and 89% in the unstimulated cultures to 9%, 2% and 48% in the stimulated cultures in the first three weeks). Downmodulation of GATA1 in STAT5-activated cells restored the balance between erythroid and myeloid differentiation as determined by MGG staining, as well as by FACS for CD11b which was retained in the cultures in which GATA1 was downregulated (from 82%, 79% and 88% in the unstimulated cultures to 86%, 79% and 81% in the 4OHT-stimulated cultures (Fig.2A) The expression of other myeloid and erythroid markers at week 2 is shown in figure 2B, and these data indicate that also the expression of CD14 and CD15 was restored to levels comparable to control groups upon downregulation of GATA1.

The STAT5-induced erythropoiesis was most pronounced within the first three weeks of MS5 coculture. Thereafter, the erythroid cells had exhausted and the STAT5A cultures displayed a more myeloid phenotype, comparable to control cultures (Fig.2A and C). At this point, the suspension cells of the unstimulated cultures were almost exclusively granulocytic, with up to 90% of the cells expressing CD15 Fig.2A and C). Regardless of GATA1 downmodulation, the activated STAT5-expressing cells displayed a clear CD11b-positive myeloid phenotype at week 5, although granulocytic maturation was less pronounced since less segmented nuclei were observed in the MGG-stained cytospins (Fig.2A). CD15 expression was slightly lower as compared to control cultures (Fig.2C).

The reversal of the STAT5-induced misbalance between erythroid and myeloid differentiation by the depletion of GATA1 was further investigated by assessing the myeloid, erythroid and megakaryocytic colony formation potential of cord blood progenitors in the presence or absence of GATA1. To obtain pure populations of transduced megakaryocytic/erythroid versus myeloid progenitors, the cord blood cells were sorted after transduction on the basis of $EGFP^+$, $CD34^+$, $CD38^+$, $CD123^+$ and $CD45RA^+$ for the GMP progenitors and $EGFP^+$, $CD34^+$, $CD38^+$ $CD123^-$ and $CD45RA^-$ for the MEP fraction. As shown in the upper two graphs in figure 2D, both

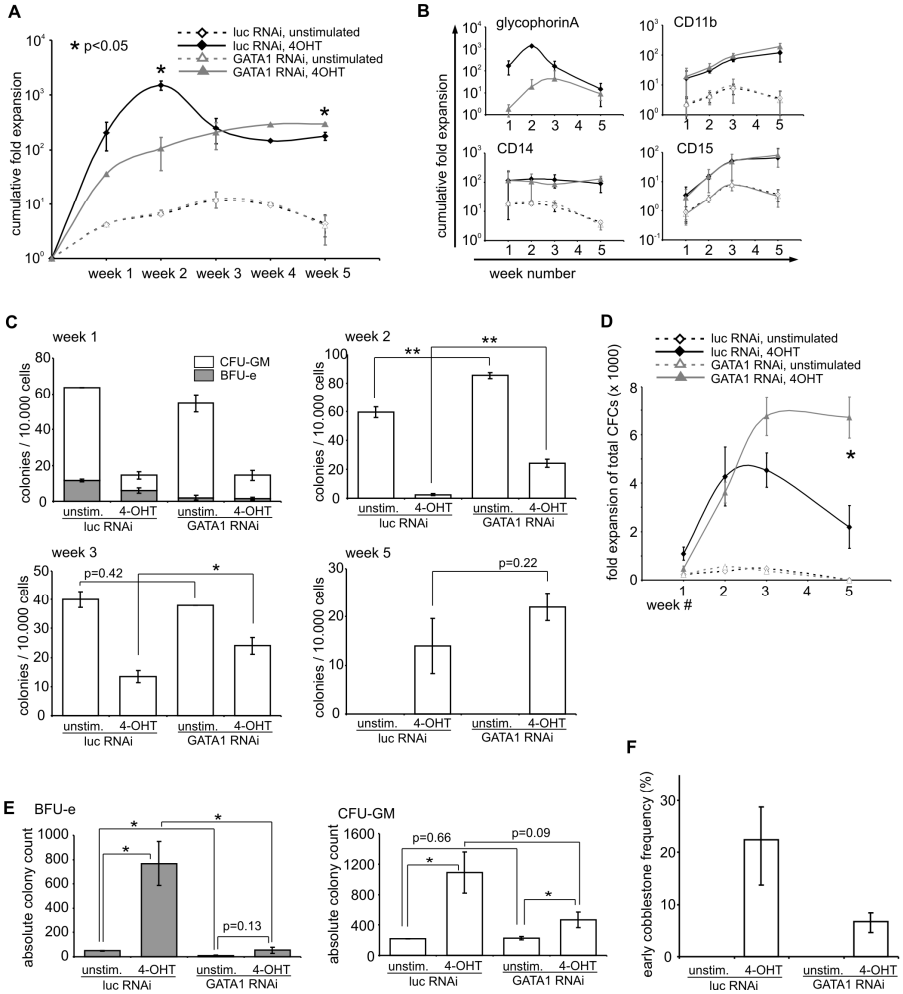

Figure 3: STAT5 induced long term growth is enhanced in the absence of GATA1. (A) CD34$^+$ cord blood cells were transduced with STAT5A-ER and the indicated lentiviral shRNA constructs. After transduction, cells were cultured on MS5 stromal cells and either left untreated or stimulated with 4-OHT. Each week, half of the medium was removed and the cells were counted and analysed by flow cytometry to calculate the number of double transduced cells. The cumulative expansion of double transduced cells was calculated relative to the starting amount of double transduced cells, which was set at 1. The mean and standard deviation of two experiments is shown, which is representative of four experiments performed. (B) The same cells as in A were stained for Glycophorin A, CD11b, CD14 and CD15. The cumulative fold expansion of the cells, separated by the respective phenotype is shown. (C) Double transduced CD34$^+$ cord blood cells as in A were sorted after 1, 2, 3 and 5 weeks and progenitor frequencies per 10.000 plated cells were determined by culture in CFC-mix methylcellulose medium. (D) Cumulative expansion of progenitors was calculated based on the progenitor frequencies depicted in C and the cumulative expansion as shown in A. (E) The absolute amount of colonies at week 1, divided by BFU-e and CFU-GM was calculated according to the expansion shown in A. (F) Early (day 10) cobblestone frequency of cordblood CD34$^+$ cells, transduced as in A and cultured in limiting dilution on MS5 stromal cells. Significant differences (two-tailed T-test) are indicated with a single ($p<0.05$) or a double asterisk ($p<0.01$).

the MEP and the GMP sorted cell populations were highly pure, resulting in 94% BFU-E and 98% CFU-GM in the respective populations. After knockdown of GATA1 however, the proportion of BFU-Es was strongly reduced from 350 to 14 per 10^3 plated cells, while the CFU-GM in the MEP fraction was increased from 23 to 173 per 10^3 plated cells, corresponding with an increase from 6% to 93% in CFU-GM colony frequency. These data suggest that the lineage fate of MEPs can be redirected into GMPs upon downmodulation of GATA1 expression. The proportion of CFU-GM colonies in the GMP fraction was not affected by GATA1 downmodulation, probably as a result of low expression levels of GATA1 in this population (shown in figure 2E). Intriguingly, the percentages of cells within the MEP and GMP gates had not yet changed within 24 hrs after transduction with GATA1 shRNA vectors.

STAT5-induced long-term growth is enhanced by GATA1 depletion

Besides differentiation, long-term expansion of hematopoietic stem/progenitor cells is markedly enhanced by expression of active STAT5 (schematically illustrated in Fig.1A).[18] Since depletion of GATA1 had profound effects on the STAT5-induced differentiation of cord blood $CD34^+$ cells, we next investigated the contribution of GATA1 on STAT5-induced long-term expansion. Cord blood $CD34^+$ cells were transduced with vectors expressing STAT5-ER in the presence of a control vector expressing luciferase shRNA or GATA1 shRNA. Cells were plated on MS5 stromal cells and proliferation in the cultures was analyzed weekly. Progenitor frequencies in the double transduced cells were analyzed by weekly CFC assays. The cumulative cell growth expressed as fold expansion and normalized with respect to the initial seeded cell numbers is shown in figure 3A. Initially, in the first two weeks, the cultures with the active STAT5 and the control shRNA proliferated more strongly compared to the GATA1-depleted cultures. After two weeks however, the cellular output of the control cultures dropped whereas the output of the cultures expressing the short hairpin against GATA1 continued to increase, leading to a reproducibly higher cellular output at week four and five. The specific expansion of erythroid (Glycophorin A) and myeloid (CD11b, CD14, CD15) cells is shown in four separate panels in figure 3B. Within the first few weeks of co-culture on MS5, it is clear that particularly the STAT5-induced erythroid differentiation was blocked by downmodulation of GATA1, while the absolute numbers of myeloid cells were not

affected at these timepoints. At week 5, no erythroid cells were observed anymore and cocultures produced predominantly myeloid cells. In activated STAT5 cocultures significantly more (myeloid) progeny was produced at week 4 and 5.
In figure 3C and D the progenitor frequency and fold expansion of progenitors in the harvested suspension fractions of the cultures at the different time points are shown. In the first three weeks, the progenitor frequency of the unstimulated cultures (with no active STAT5) was higher than in the cultures with active STAT5 as determined by the number of CFCs per 10^4 plated cells (Fig.3C). Since the total number of cells was strongly increased upon STAT5 activation, the total expansion of CFCs in STAT5 cultures was strongly increased as compared to control cultures (Fig.3D). At week 1, downmodulation of GATA1 significantly impaired the total amounts of BFU-Es that were produced, both in control as well as in STAT5-transduced cultures, while GATA1 downmodulation did not significantly change total CFU-GM numbers (Figure 3E). At week 5 the unstimulated cultures were depleted of any progenitor activity, whereas the cultures with active STAT5 were still producing progenitors (in line with previously obtained results[18]).
A striking feature of active STAT5 expressing cells is the potential to form cobblestone areas underneath MS5 bone marrow stromal cells. To enumerate the proportion of early cobblestone forming cells, cord blood $CD34^+$ cells were similarly transduced as in panel A and were seeded in limiting dilution on MS5 stromal cells in 96 well plates. After ten days, the wells positive for cobblestone formation were scored and the frequency was calculated. Figure 3F shows that approximately 1 in 5 plated cells (22%) formed early cobblestones in the presence of active STAT5, which was, although reduced, not abrogated in the absence of GATA1. Furthermore, after five weeks of culture, cobblestones were still present in both cultures with active STAT5, regardless of the presence of GATA1 (data not shown).

Identification of GATA1-dependent and GATA1-independent STAT5 target genes

Since depletion of GATA1 from $CD34^+$ cord blood cells could dissect the phenotypes caused by STAT5 activation (differentiation vs. long term proliferation), the underlying molecular mechanisms were investigated at the level of gene expression. $CD34^+$ cord blood cells were transduced with conditionally active STAT5 in the presence or absence of short hairpins against GATA1. After transduction, the transduced cells were cultured for three days in serum-free

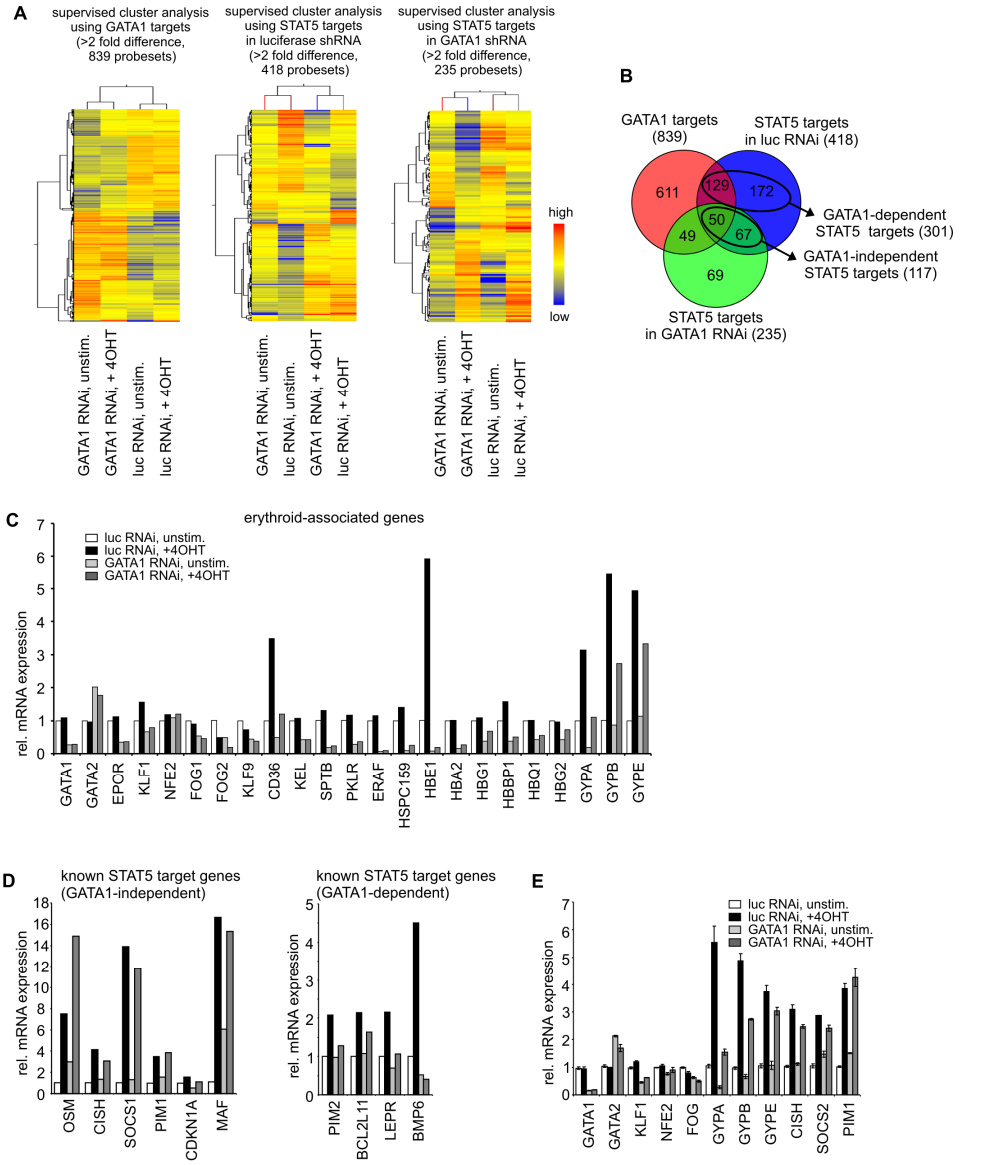

Figure 4: STAT5 induced genes can be divided in GATA1 dependent and GATA1 independent. (A) Cordblood CD34$^+$ cells, transduced with 4-OHT inducible STAT5 and lentiviral vectors expressing short hairpins against luciferase and GATA1 were cultured for three days, STAT5 was induced by 4-OHT stimulation for 24 hours and RNA was extracted. Equal amounts of RNA from three independent performed experiments was pooled and hybridized to Illumina arrays. Supervised cluster analysis of, left panel: GATA1 targets with more then two fold difference, middle panel: STAT5 target genes in luciferase shRNA transduced samples and right panel: STAT5 target genes in GATA1 shRNA transduced samples. (B) Venn diagram showing differences and overlap between the three groups analyzed in A. (C) Array-derived gene expression data of known erythroid associated genes. (D) Array-derived gene expression data of a number of previously described STAT5 target genes, divided as GATA1-dependent and GATA1-independent STAT5 target genes. (E) Q-RT-PCR verification of a number of genes shown in C and D.

medium and either left untreated or stimulated with 4-OHT to induce STAT5 activity for 24 hours, after which the double transduced cells were sorted and total RNA was extracted. Equal amounts of RNA of three independent experiments were pooled, reverse transcribed and hybridized to human Illumina BeadChip Arrays that contained 46k probesets. 839 probesets were differentially expressed between the luciferase and GATA1 short hairpin-transduced groups (Supplemental Table 1) and a supervised cluster analysis of these GATA1 targets is shown in figure 4A. 434 genes were downregulated upon GATA1 depletion, and GO annotation[35,36] revealed that this list was highly enriched for erythroid-associated genes (Supplemental Table 1). Apparently, many of these erythroid genes are under direct control of GATA1, including EPOR, FOG1, CD36, ERAF, various hemoglobins and GYPA. 397 genes were upregulated upon GATA1 depletion, and GO annotation revealed that this list was enriched for, amongst others, genes associated with the plasma membrane, leukocyte activation and apoptosis (Supplemental Table 1). Although CD33 was also upregulated upon GATA1 depletion, no immediate upregulation of a general myeloid gene expression program was observed within 24 hrs after GATA1 downmodulation. A potentially important gene, GATA2, was upregulated approximately two-fold by GATA1 downmodulation, confirming the known reciprocal regulation of these genes.[37] The key regulators of myeloid commitment C/EBPα and PU.1 were upregulated upon downmodulation of GATA1, but the fold change did not yet reach the 2-fold significance level within the time-frame of our analyses (1.5 and 1.8-fold upregulated, respectively). Furthermore, when analyzing STAT5 target genes, it was observed that in the presence of GATA1 418 different probesets were induced by STAT5 activation, whereas 235 probesets were induced in the absence of GATA1 (supervised cluster analyses are shown in Fig.4A and data is available in Supplemental Table 2 and 3). These results led to the identification of 301 GATA1-dependent STAT5 target genes (defined as genes upregulated more than two-fold in the presence and less than two-fold in the absence of GATA1) and 117 GATA1-independent STAT5 target genes (defined as genes upregulated more than two fold irrespective of the presence of GATA1), as shown in the Venn diagram in figure 4B and Supplemental Table 4 and 5. GO annotation revealed that the GATA1-independent upregulated STAT5 targets were enriched for genes associated with growth regulation and the JAK-STAT pathway, and included a number of well described STAT5 target genes including OSM, PIM1 and CISH. Interestingly, a number of erythroid-associated genes were also upregulated by STAT5 in the absence of GATA1, including CD36, HBE1, GYPA, GYPB and

GYPE. The GATA1-dependent STAT5 targets were enriched for genes associated with signal transduction and cell proliferation, but surprisingly no erythroid-associated genes were identified that were upregulated by STAT5 in a GATA1-dependent manner. The Illumina expression data of a number of known erythroid-associated and known STAT5 target genes, divided in GATA1-dependent and GATA1-independent, is shown in figures 4C and 4D, and the expression of several of these target genes was verified by Q-PCR analysis (Fig.4E).

Discussion

We have shown previously that overexpression of (conditionally) active STAT5A in human CB-derived $CD34^+$ cells results in at least two clearly distinguishable phenotypes in MS5 bone marrow stromal cocultures. Firstly, a pronounced erythroid differentiation is induced within the first two weeks upon activation of STAT5A. Secondly, long-term proliferation is induced, which is associated with the formation of cobblestone areas underneath the stroma capable of self-renewal as determined by their long-term serial replating capacity.[18,19,21] In the present study, we aimed to dissect these different phenotypes and identified that the differentiation phenotypes imposed on cord blood $CD34^+$ cells by active STAT5A could be uncoupled from the long term growth phenotype by downregulation of GATA1.

It remains unknown how activation of STAT5A can contribute to the induction of erythropoiesis. Mouse embryos that lack STAT5 expression are severely anemic, due to enhanced apoptosis as a consequence of reduced expression levels of the anti-apoptotic proteins Mcl1 and Bcl-XL.[10,22] However, within the conditions of our assays we found no evidence that these genes were strongly upregulated by activation of STAT5. Furthermore, STAT5-deficient cells display reduced levels of IRP-2/IREB2, which regulates the translation of TfR1,[22] but within the time-frame of our gene expression studies we also did not find evidence that IREB2 expression levels are enhanced by activated STAT5. Because GATA1 is well known for its role in erythropoiesis,[26,31] the role of this protein in the STAT5-induced phenotypes was investigated in the present study. GATA1 expression was downregulated using a lentiviral RNAi approach, and our data clearly indicate that GATA1 is required for STAT5-induced erythropoiesis. This result is in line with the present understanding that GATA1 is essential for erythropoiesis, but does highlight that GATA1 is not dispensable for STAT5-induced erythroid commitment. We tried to address whether the STAT5-induced signals involved in erythroid commitment were all mediated though GATA1, or whether STAT5 and GATA1 could also act independently. We found no evidence that STAT5 could upregulate the expression of GATA1 in CB $CD34^+$ cells and thereby drive erythropoiesis (Fig. 1 and 4), and no experimental evidence was obtained that STAT5 overexpression enhances GATA1 transcriptional activity as determined in α-globin promoter-driven luciferase reporter assays (unpublished observations). Previously, we did observe that overexpression of another activating mutant of STAT5, STAT5A(1*6), did slightly

enhance GATA1 expression levels in mobilized PB CD34$^+$ cells.[38] Whether differences in cell type or in levels of STAT5 activity underlie these observations is currently unclear, but also in a previous study in which we studied STAT5 target gene expression in relation to the dosage of STAT5 activity using the STAT5-ER vectors we did not observe any effects on GATA1 expression.[19] Whether post translational modifications like acetylation of GATA1[39] could play a role in the STAT5-induced erythroid differentiation is at present unknown. To get more insight into the interplay between STAT5 and GATA1 in the process of erythroid commitment, we performed expression array analysis on CB CD34$^+$ cells transduced with a conditional expression vector for STAT5 in the absence and presence of GATA1. A number of conclusions can be drawn from these experiments. Firstly, as expected, various erythroid-associated genes were downregulated upon depletion of GATA1, including EPOR, KLF1, FOG1, FOG2, CD36, ERAF and several hemoglobin genes. These data are in line with previously published data.[40] Interestingly, a number of these erythroid-associated genes can also be activated by STAT5, even in the absence of GATA1, including CD36, HBE1 and Glycophorin A. Apparently, STAT5 and GATA1 can act on these promoters independently, and we indeed indentified STAT5 response elements in their promoter sequences (data not shown). But since no erythroid commitment was observed in the absence of GATA1 it can be concluded that the STAT5-induced expression of these genes by itself is not sufficient for erythroid development. We also identified genes that were induced by STAT5 in a GATA1-dependent manner (Supplemental Table 5), suggesting that STAT5 and GATA1 can cooperatively act on a number of genes as well. However, no genes were identified in this list that has directly been linked to erythroid development. Gene ontology analysis revealed that this group is enriched for genes associated with signal transduction, cell communication and proliferation, and future studies will clarify the role of these genes in STAT5-induced erythroid differentiation.

It has been well documented that key regulators of myeloid/erythroid cell fate decisions such as PU.1, C/EBPα and GATA1 are capable of directly affecting each others activities.[41-43] For instance, once erythroid commitment is initiated an increase in GATA1 expression or activation levels will immediately impose negative feedback control on the myeloid transcription factors by negatively affecting their transcriptional activities and ultimately also their expression levels. We observed that downregulation of GATA1 in the megakaryocyte/erythroid progenitor (MEP) compartment not only impaired BFU-E formation, but also resulted in increased frequencies of CFU-GM progenitors (Fig.2D). Although our cloning efficiencies do

not exceed 35% in these assays and therefore single cell assays are required to study erythroid-to-myeloid lineage switching, our data do suggest that downmodulation of GATA1 is sufficient to convert BFU-E into CFU-GM progenitors. In line with these data, we find that downmodulation of GATA1 expression is sufficient to enhance expression of C/EBPα and PU.1, although the fold change did not yet reach the 2-fold significance level within the time-frame of our analyses (1.5 and 1.8-fold upregulated, respectively).

Another marked feature of persistent STAT5 activation is the enhanced proliferation potential of stem and progenitor cells.[18,19] The effective knockdown of GATA1 expression, thereby preventing erythropoiesis, provided us with the opportunity to study the effects of STAT5 over expression in the absence of erythroid differentiation. Within the first two weeks of MS5 coculture the proliferative advantage of STAT5 cells over controls was less dramatic in the absence of GATA1 as compared to when GATA1 was present. After three weeks of culturing, the number of suspension cells as well as the number of progenitors in STAT5 cultures that were generated in the absence of GATA1 exceeded those that were generated in the presence of GATA1. Apparently, STAT5-induced erythroid differentiation resulted in exhaustion of the stem cell/progenitor pool, and inhibition of erythroid commitment by GATA1 downmodulation allowed a better long-term expansion and stem cell/progenitor maintenance. These data are in line with our previously published transcription factor dosage studies whereby we observed that intermediate STAT5 activity levels resulted in maximal long-term expansion, while high STAT5 activity levels resulted predominantly in erythroid commitment and exhaustion of the stem cell/progenitor compartment.[19] As observed previously, early cobblestone formation was clearly enhanced in the presence of active STAT5, but the frequency was approximately threefold downregulated in the absence of GATA1, despite the capacity to generate long-term expanding cultures under those conditions. Apparently, the 10-day cobblestones represent a mixture of erythroid predisposed progenitor cells that lack long-term self-renewal capacity as well as cobblestone areas that represent more primitive cells that retain the capacity for long-term self-renewal and can sustain STAT5-induced long-term expansion for over 5 weeks. Another gene which was upregulated after GATA1 depletion was GATA2, confirming the known reciprocal regulation of these genes.[37] GATA2 is known to be involved in regulation of stem cell quiescence, with high expression levels in the most quiescent stem cells.[27] GATA2$^{-/+}$ mice are described to have impaired competitive repopulation potential, accompanied by reduced CFC and LTC-IC frequencies.[44] Possibly, increased GATA2 in conjunction with the

elevated expression of C/EBPα and PU.1 in the cells transduced with GATA1 shRNA in the presence of active STAT5 facilitates further expansion of these cells. However, GATA2 upregulation in the control cultures did not result in increased long term proliferation. Also, overexpression of GATA2 in human stem and progenitor cells has been described to result in quiescence and impaired function of these cells,[45] so whether the degree of upregulation of GATA2 is the cause of the increased long term output in our setting is not clear.

The uncoupling of the STAT5-induced phenotypes with the erythroid lineage provided a number of interesting GATA1-independent STAT5 target genes. GO annotation revealed that the GATA1-independent upregulated STAT5 targets were enriched for genes associated with growth regulation and the JAK-STAT pathway. Indeed, growth related genes like PIM1 and MAF were represented in this group. PIM1 is well known for its role in the response to hematopoietic growth factors[46,47]. PIM1 might also play a role in the enhanced interaction between STAT5-expresing stem/progenitor cells and the formation of cobblestone areas since it was recently shown that FLT3-ITD-induced leukemogenesis depends on PIM1 expression to regulate CXCL12-CXCR4-mediated homing and migration.[48] MAF is an important transcription factor involved in many different cell types, including myeloid hematopoietic cells.[49] In Multiple Myeloma, the oncogene MAF has been shown to stimulate cell cycle progression as well as to improve the interaction between tumor and stromal cells.[50] The dissection of the precise roles that these genes fulfill in the process of STAT5-induced long-term stem cell/progenitor expansion is under current investigation.

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Supplemental data (available upon request)

Supplemental Table 1: GATA1 target genes in CB $CD34^+$ cells

Supplemental Table 2: STAT5 target genes in shLuciferase-transduced CB $CD34^+$ cells

Supplemental Table 3: STAT5 targets genes in shGATA1-transduced CB $CD34^+$ cells

Supplemental Table 4: GATA1-dependent STAT5 targets genes in transduced CB $CD34^+$ cells

Supplemental Table 5: GATA1-independent STAT5 targets genes in transduced CB $CD34^+$ cells

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